Evidence for unidirectional flow through plasmodesmata

Evidence for Unidirectional Flow through Plasmodesmata[1]

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The leaf trichome of tobacco (*Nicotiana tabacum*) represents a unique secretory structure in which the basal trichome cell is connected to the epidermis by numerous plasmodesmata (PD). Small fluorescent probes microinjected into the basal trichome cell moved apically into distal trichome cells but not into the subtending epidermal cell. In marked contrast, the same probes moved apically into trichome cells when injected into the epidermal cell. Noninvasive methods of dye loading, including ester loading into the apical secretory cell by trichome "capping" and by infiltration of caged fluorescein, produced the same result. In transgenic tobacco plants constitutively expressing photoactivatable green fluorescent protein (PAGFP), activation of PAGFP above the epidermal/trichome (e/t) boundary resulted in movement of protein apically into the distal trichome cells but not across the e/t boundary, while PAGFP activated in the epidermal cell moved apically across the e/t boundary. Experiments with apoplastic tracers also revealed the presence of a distinct apoplastic barrier to solute movement at the e/t interface. These data point to unidirectional transport of solutes through PD. PAGFP activated in individual cells equidistant between the basal cell and the apical cell moved bidirectionally from these cells, suggesting that mass flow was not the driving force for unidirectional transport. We found that unidirectional transport across the e/t boundary was not affected by virus infection or by addition of the actin inhibitor latrunculin but could be dissipated completely by addition of sodium azide. Collectively, our data suggest that active, unidirectional transport of molecules may occur through PD located at unique interfaces in the plant.

Plasmodesmata (PD) are the functional units of the symplasm and occur at multiple interfaces between plant cells (Roberts, 2005; Maule, 2008). The basic structure of the PD pore reveals a plasma membrane (PM)-lined pore containing the desmotubule, an axial endoplasmic reticulum-derived structure that provides endomembrane continuity between cells (for review, see Maule, 2008). The space between the desmotubule and the PM is known as the cytoplasmic sleeve and is thought to be the main conduit for the movement of small solutes between cells (Erwee and Goodwin, 1983; Terry and Robards, 1987; Liarzi and Epel, 2005). PD have received considerable attention in recent years for their ability to traffic large macromolecules, including proteins and nucleic acids (Lucas and Lee, 2004; Maule, 2008). For many macromolecules, transport through the PD pore appears to occur by a selective mechanism that permits an exchange of macromolecules between specific cell types (Wu et al., 2002; Lucas and Lee, 2004; Zambryski, 2004).

For small solutes, movement through the PD pore is thought to occur by diffusion and is strongly dependent on the hydrodynamic, or Stokes, radius of the permeant molecule in question (Liarzi and Epel, 2005). The effective channel for solute transport through PD is in the region of 3 nm (Erwee and Goodwin, 1983; Terry and Robards, 1987; Tucker, 1988), and molecules of less than 1 kD are thought to pass freely between cells (Erwee and Goodwin, 1983; Terry and Robards, 1987). However, a recalculation of the data presented by Terry and Robards (1987) by Fisher (1999) revealed that the effective diameter for transport through the cytoplasmic sleeve may be closer to 4 nm, potentially allowing significantly larger molecules to pass through the pore. Indeed, GFP (27 kD, Stokes radius of 2.8; Liarzi and Epel, 2005) may move diffusively between PD of some cell types, depending on tissue location and age (Oparka et al., 1999; Crawford and Zambryski, 2001; Wymer et al., 2001; Liarzi and Epel, 2005). Although the size exclusion limit of the PD pore was originally thought to be relatively constant (Erwee and Goodwin, 1983; Terry and Robards, 1987), it is now clear that "not all PD are equal," in the sense that the size exclusion limit (SEL) of the pore may differ substantially during cell development (Duckett et al., 1994; Oparka et al., 1999; Crawford and Zambryski, 2000; Ruan et al., 2001) in response to environmental conditions (Epel and Erlanger, 1991; Cleland et al., 1994; Schulz, 1995) and the location of a given cell-cell interface in the plant (Erwee and Goodwin, 1983, 1985;

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Duckett et al., 1994; Ruan et al., 2001). It is also established that the SEL of the pore may respond to intracellular cues such as water relations (Oparka and Prior, 1992; Schulz, 1995), metabolic status (Tucker, 1993; Cleland et al., 1994; Wright and Oparka, 1997), cytosolic Ca^{2+} levels (Tucker, 1988; Tucker and Boss, 1996; Holdaway-Clarke et al., 2000), and hormones (Ormenese et al., 2006). The picture emerging is one in which the PD pore functions as a dynamic structure that alters its SEL in response to changing local conditions (Maule, 2008). Despite this, simple diffusion remains the most likely mechanism determining solute exchange between symplasmically coupled cells (Liarzi and Epel, 2005).

A common feature in plant tissues is the presence of symplasmic "domains" within which PD share a common SEL (for review, see Roberts and Oparka, 2003). Examples of symplasmic domains occur at interfaces where cells of differing functions are connected by PD. In extreme cases, such as guard cells, PD are lost during development from the guard cell walls that connect the stomatal complex to the epidermis, effectively isolating the guard cells from the epidermal symplasm (Palevitz and Hepler, 1985). Commonly, however, cells of differing functions remain connected by PD, although these interfaces appear to be tightly regulated with respect to solute flow (Roberts and Oparka, 2003). An example of strict control occurs in the PD that connect the sieve element-companion cell complex with mesophyll cells (van Bel and van Rijen, 1994; Knoblauch and van Bel, 1998; Oparka and Turgeon, 1999). These PD appear to be closed during normal long-distance phloem functions (van Bel and van Rijen, 1994; Knoblauch and van Bel, 1998; Oparka and Turgeon, 1999) but may open in response to stress conditions (Schulz, 1995; Wright and Oparka, 1997). For those differing cell types that remain connected by PD, a major challenge is to retain cell autonomous functions in the presence of symplasmic connections.

Recently, we made an extensive study of the interface that connects the basal leaf trichome cell of tobacco (*Nicotiana tabacum*) with the underlying epidermal cell (Faulkner et al., 2008). A simple freeze-fracture protocol allowed PD to be viewed in surface fractures of the cell wall and PM, and we were able to image all of the PD across the cell-cell interface of the epidermis/trichome (e/t) boundary. Our data showed that during wall development, simple primary PD were gradually replaced with secondary PD by a multiple twinning process that involved the de novo formation of PD pores. During trichome development, the number of PD at the e/t boundary increased 5-fold due to the insertion of new secondary PD pores. In mature trichomes, in excess of 2,000 PD were found in the cell wall spanning the e/t boundary. The ability to image large numbers of PD at this interface prompted us to explore more closely the function of PD at the e/t interface. Previously, trichomes have proved a useful experimental system for studying cell-cell transport through PD (Waigmann and Zambryski, 2000). Interestingly, trichome cells of *Nicotiana clevelandii* have been shown to be connected by PD with a large basal SEL of about 7 kD (Waigmann and Zambryski, 1995) and appear to differ in structure from mesophyll PD (Waigmann et al., 1997). However, the specific interface between the trichome basal cell and the epidermis has not been examined.

To examine the function of PD at the e/t interface in more detail, we developed a number of different approaches for introducing fluorescent probes into the symplasm of the basal trichome cell and its supporting epidermal cell. These included microinjection, cell-specific dye loading, the use of caged fluorescein, and the generation of transgenic tobacco plants constitutively expressing photoactivatable GFP (PAGFP) in the cytosol. Our data show consistently that fluorescent probes introduced into the basal trichome cell are unable to cross the e/t boundary while probes introduced below this boundary are able to enter the trichome freely, providing strong evidence for unidirectional transport through PD at a specific cell-cell interface. In addition, we show that sodium azide, a metabolic inhibitor that alters PD SEL (Tucker, 1993), dissipates unidirectional solute flow into the trichome and allows molecules to exit the trichome. Our data are consistent with unidirectional solute movement into the basal trichome cell.

RESULTS

Microinjection

In our initial experiments, we microinjected a range of fluorescent probes (fluorescein, Lucifer yellow CH [LYCH], sulforhodamine G) into the basal trichome cell to determine symplasmic continuity between the basal trichome cell and the underlying epidermis (Fig. 1A). As all dyes gave the same result, we present here data for the most membrane impermeant of these, LYCH. Given the large numbers of PD at the e/t interface (Faulkner et al., 2008), we were surprised that in 35 of 39 injections, LYCH failed to move across the e/t boundary and enter the epidermis (Fig. 1A). However, in each injection, LYCH moved apically into other trichome cells (Fig. 1A). In all cases, cytoplasmic streaming resumed immediately after dye injection. When dye was observed to have crossed the e/t barrier, only extremely weak fluorescence was observed in the underlying epidermal cell. In contrast, if we injected LYCH into the supporting epidermal cell, dye moved apically across the e/t boundary into the trichome (Fig. 1B). With time (1–3 h), all dyes were sequestered slowly into the central vacuoles of the trichome cells, sulforhodamine G showing the highest rate of vacuolar sequestration (data not shown).

Symplasmic Transport of PAGFP

We used a transgenic tobacco line constitutively expressing cytosolic PAGFP to assess the capacity for

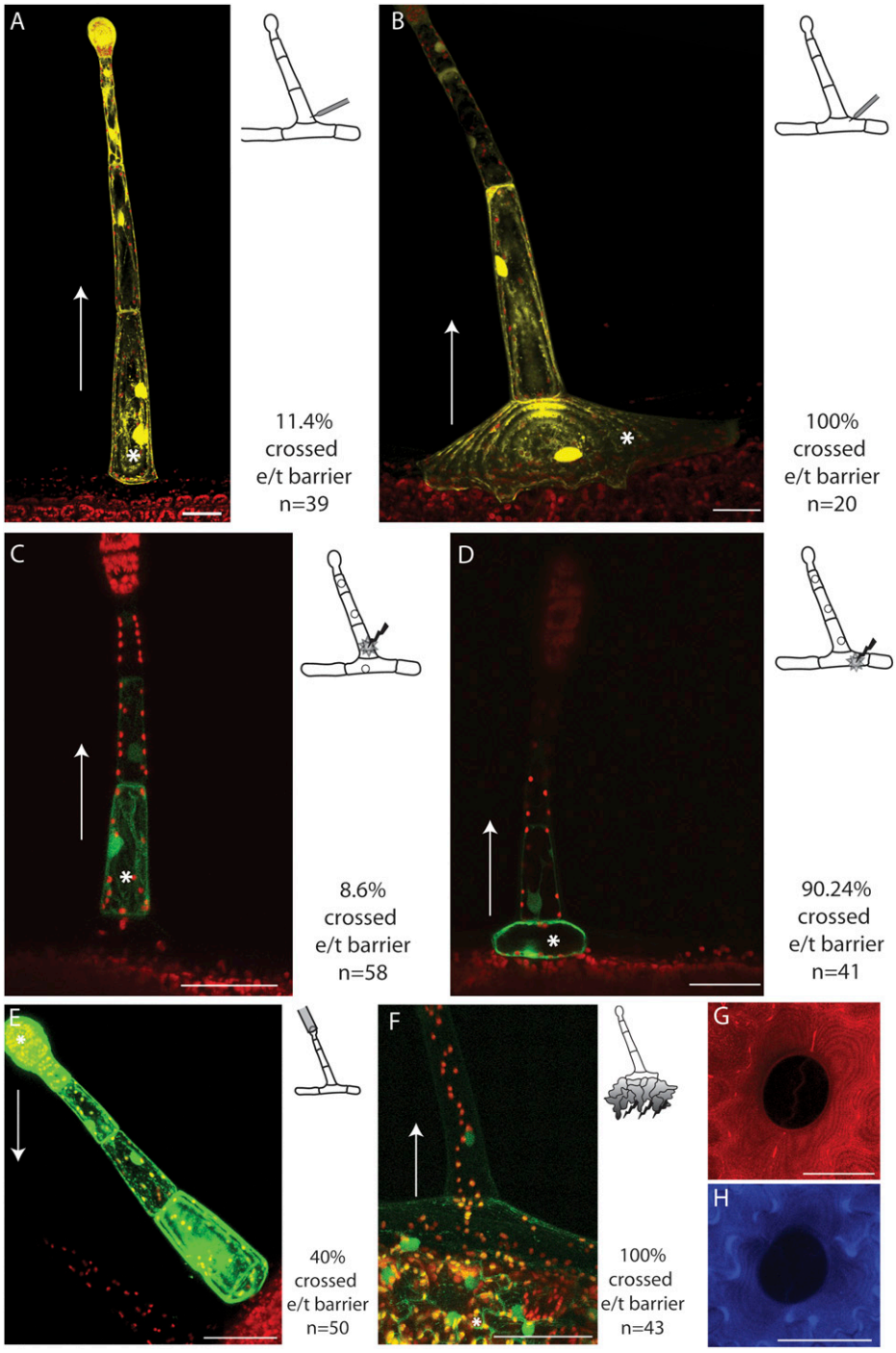

Figure 1. Introduction of fluorescent probes into the symplasm of tobacco leaf trichomes identifies a symplastic and an apoplastic barrier at the e/t interface. Cells into which probes were introduced are marked by asterisks, while data relating to each treatment are shown to the right of each image and arrows show the direction of the flow. Illustrations showing the method of tracer delivery are shown to the right of each image. A, Microinjection of LYCH into the basal trichome cell showing apical movement into cells of the trichome chain. Bar = 40 μm. B, Microinjection into the supporting epidermal cell showing apical movement into the leaf trichome. Bar = 50 μm. C, GFP, activated from PAGFP in the basal trichome cell, moves apically into other trichome cells. Bar = 50 μm. D, GFP, activated from PAGFP in the epidermal cell, moves apically into the leaf trichome. Bar = 40 μm. E, Capping of the apical secretory cell with FDA shows movement of the ester-loaded fluorescein basipetally down the trichome. Bar = 50 μm. F, Caged fluorescein, activated by UV light in the mesophyll, moves apically into the leaf trichomes. Bar = 75 μm. G, Infiltration of the apoplast with propidium iodide (red) shows the absence of staining of the trichome basal cell wall. The image is derived from an optical stack taken vertically down through the trichome-epidermis boundary, revealing the cell wall as an unstained disc. Bar = 50 μm. H, As for G, using Calcofluor white as the apoplastic probe. Bar = 50 μm.

symplasmic protein transport in the trichome. A region of cytoplasm containing the basal cell nucleus was exposed to a brief pulse of 405-nm light to photoactivate GFP in the cytosol (Fig. 1C). GFP moved rapidly through the cytoplasm of the photoactivated cell and within 3 h had moved apically into additional trichome cells in 100% of activations (Fig. 1C). GFP did not move across the e/t boundary in 91.4% of activations. We then photoactivated GFP in the supporting epidermal cell, taking care to photoactivate a region distant from the e/t boundary with 405-nm radiation (Fig. 1D). In this case, GFP crossed the e/t boundary and entered the basal cell of the trichome in 90% of activations (Fig. 1D).

Capping of Trichomes

We considered the possibility that microinjection of the basal trichome cell may have caused a pressure differential sufficient to close PD at the e/t boundary

(Oparka and Prior, 1992). Therefore, we sought alternative ways of introducing dyes into the symplasm. The most distal (apical) cell of the trichome is secretory and releases exudate through transient pores in the outer cuticle (Waigmann and Zambryski, 2000). We exploited this pathway to ester load dyes into the apical cell cytoplasm. The apical cell of the trichome was "capped" with a microcapillary containing the membrane-permeant probe fluorescein diacetate (FDA). Once in the cytosol, the nonfluorescent form of the dye was cleaved by intracellular esterases to release the membrane-impermeant fluorescein moiety. Fluorescein moved rapidly down the chain of trichome cells in the symplast and was restricted at the e/t boundary in 60% of trichomes (Fig. 1E). When fluorescein crossed the e/t boundary, the fluorescence in the epidermal cell was detected at very low levels.

Symplasmic Transport of Uncaged Fluorescein

In order to load fluorescein noninvasively below the e/t boundary, we infiltrated leaf tissue with membrane-permeant "caged" fluorescein (Martens et al., 2004). We then uncaged the fluorescein moiety by exposing the leaf tissue subtending the trichomes to a brief (2–4 s) pulse of UV light (Fig. 1F). This UV pulse immediately uncaged the fluorescein moiety, which subsequently moved symplasmically into the leaf epidermis and into all of the trichomes at the leaf margin (Fig. 1F).

An Apoplastic Barrier Is Present at the e/t Boundary

In their work on the *Abutilon* secretory trichome, Gunning and Hughes (1976) noted an apoplastic barrier at the basal ("stalk") cell of the trichome that restricted apoplastic transport of the cell wall probe, Calcofluor white, into the trichome apoplast. To determine whether a similar barrier was present at the base of the tobacco leaf trichome, we infiltrated the apoplastic markers Calcofluor white and propidium iodide into the apoplast below the leaf margin. These probes moved freely in the mesophyll and epidermis apoplast but did not move along the basal trichome walls. Optical sections taken parallel to the leaf surface revealed clearly that these probes had not stained the cell wall at the e/t boundary (Fig. 1, G and H).

Inhibitor Studies

To determine whether the unidirectional movement of dyes could be reversed, we infiltrated inhibitors into the leaf margin. Specifically, we examined two inhibitors, latrunculin (Ding et al., 1996) and sodium azide (Tucker, 1993), that have been shown previously to increase the basal SEL of PD in plant tissues. Application of latrunculin arrested cytoplasmic streaming in the basal trichome cell and also disrupted the actin cytoskeleton (data not shown). However, LYCH injected into the basal trichome cytoplasm failed to move across the e/t boundary in the presence of latrunculin (data not shown; $n = 20$). In marked contrast, when we applied sodium azide to the leaf margin, injected LYCH moved freely across the e/t boundary and moved symplasmically into all cells of the epidermis and mesophyll in 90% of injections. In addition, LYCH moved along the leaf margin and apically into other noninjected trichome cells (Fig. 2A).

The Presence of Viral Movement Protein Does Not Facilitate Dye Transport across the e/t Boundary

Previously, it was shown that transgenic expression of the tobacco mosaic virus (TMV) movement protein (MP) increases the SEL of tobacco mesophyll PD (Wolf et al., 1989). To determine whether MP could influence the e/t barrier, we microinjected LYCH into trichomes of a tobacco line that expressed a functional MP-GFP fusion (Roberts et al., 2001; Faulkner et al., 2008). MP failed to influence the PD at the e/t boundary, and dye remained within the injected basal trichome cell in 90% of injections (Fig. 2B). In addition, we capped trichomes of the MP-GFP line with FDA, as described above for wild-type plants. As before, fluorescein moved down the chain of trichome cells but did not cross the e/t boundary in 70% of the injections (data not shown; $n = 51$). We also examined the e/t barrier in plants expressing PAGFP when the tissue was infected with TMV expressing the fluorescent reporter DsRed (Christensen et al., 2009). GFP did not move across the e/t barrier when activated in the basal cell of virus-infected trichomes in 98% of activations ($n = 55$). Furthermore, in these trichomes, GFP moved apically into other trichome cells in only 26% of activations, suggesting restricted apical movement through PD (data not shown).

The e/t Symplasmic Boundary Is Established Early in Trichome Development

We used PAGFP-expressing plants to examine the development of the e/t boundary. Photoactivation of GFP in immature, single-celled trichomes arising from the leaf epidermis (Faulkner et al., 2008) resulted in no GFP movement into subtending epidermal cells in 52% of activations (Fig. 2C), suggesting that the e/t boundary is established at the basal trichome wall early in trichome development. When movement of GFP occurred across the e/t barrier, it was observed within 1 h of activation.

Pressure Probe Measurements

To examine whether the basal trichome cell had a different turgor pressure from the supporting epidermal cell, we used the pressure microinjector to measure cell turgor pressure as described previously (Oparka et al., 1991; Oparka and Prior, 1992). There was no significant difference ($P = 0.13$ by two-tailed t test) in the turgor pressure of the basal trichome cell

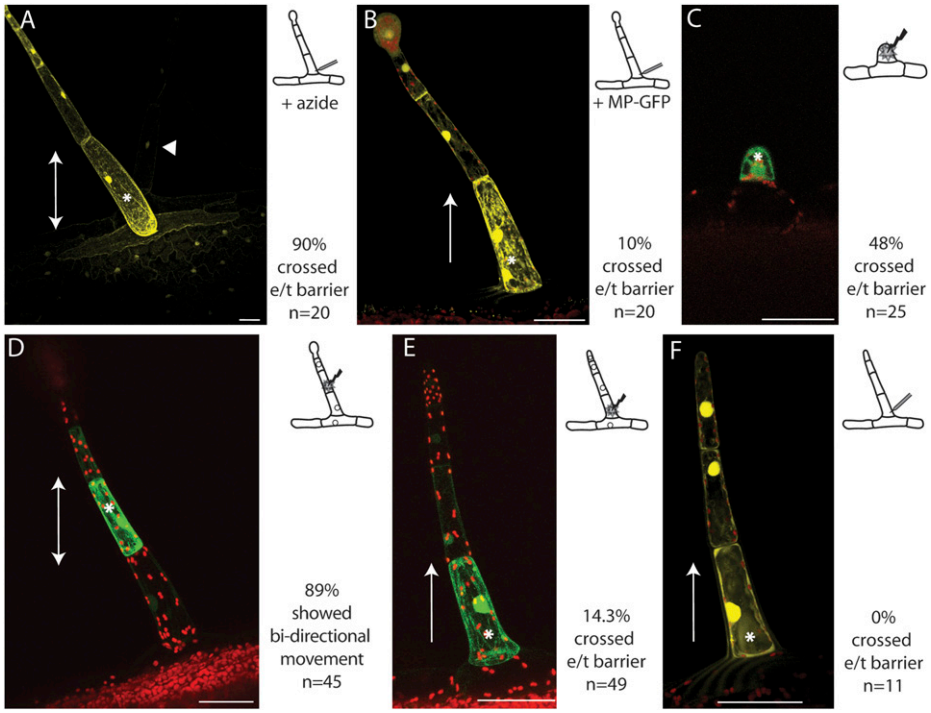

Figure 2. Introduction of fluorescent probes into secretory and nonsecretory trichome cells in the presence of sodium azide and the TMV MP. Cells into which probes were introduced are marked by asterisks, while data relating to each treatment are shown to the right of each image and arrows show the direction of the flow. Again, illustrations of the method of delivery of the fluorescent tracers are shown to the right of each image. A, Microinjection of LYCH into the basal trichome cell in the presence of sodium azide. The dye moves apically into the trichome cells and also basally into the epidermis and mesophyll. The dye also enters adjacent trichomes from the epidermis (arrowhead). Bar = 50 μm. B, LYCH injected into the basal trichome cell of a transgenic line expressing a MP-GFP fusion moves apically into the trichome cells. Bar = 50 μm. C, Photoactivated GFP in a developing trichome initial remains in the activated cell. GFP moved to the epidermis from approximately 50% of immature, one-celled trichomes. Bar = 20 μm. D, Photoactivated GFP in cells located midway along the trichome chain shows bidirectional movement. Bar = 40 μm. E, GFP photoactivated in the basal trichome cell moves apically in nonsecretory trichomes. Bar = 40 μm. F, As for E, following injection of LYCH into the basal trichome cell. Bar = 40 μm.

(566 ± 6.8 kPa; n = 32) compared with the epidermal supporting cell (533 ± 5.6 kPa; n = 34). Thus, there appears not to be a natural turgor gradient between these cells that might explain the unidirectional transport across the e/t boundary.

LYCH and GFP Move Bidirectionally between Adjacent Trichome Cells

Given the secretory functions of trichome cells, we next asked whether the mass flow of solutes might be occurring through the chain of trichome cells, giving rise to the unidirectional movement we observed across the e/t boundary. Therefore, we activated PAGFP in single trichome cells located equidistant between the e/t boundary and the apical secretory cell. PAGFP moved bidirectionally between trichome cells (Fig. 2D), indicating a large SEL between adjoining trichome cells. Similarly, injections into these cells moved bidirectionally, at equal rates, into other trichome cells (data not shown). These data appear to negate a mass flow of solutes along the length of the trichome.

The e/t Boundary Is Also Present in Nonsecreting Trichomes

In addition to secretory trichomes, tobacco leaves also contain a population of nonsecretory trichomes that end in a pointed cell (Faulkner et al., 2008) and a further population of small prostrate trichomes involved in defense (Shepherd et al., 2005; Faulkner et al., 2008). The nonsecretory trichomes proved difficult to cap with FDA due to the lack of breaks in the trichome cuticle (data not shown). However, PAGFP failed to cross the e/t barrier when activated in the basal trichome cell of nonsecretory trichomes (Fig. 2E; 86.7% of trichomes), as did injected LYCH (Fig. 2F; 100% of trichomes). Finally, uncaged fluorescein moved freely from the mesophyll cells into all trichome cells, regardless of their function (data not shown), indicating that the e/t boundary is similar in both secreting and nonsecreting trichomes.

DISCUSSION

Solute movement through the cytoplasmic sleeve of PD is thought to occur by diffusion and is limited by the functional SEL of the pore (Liarzi and Epel, 2005). Fluorescent probe movement is generally observed within a single symplasmic domain such as the mesophyll (Wolf et al., 1989) or the epidermis (Oparka et al., 1997; Martens et al., 2004). These studies tend to show uniform radial dye movement into surrounding cells (Liarzi and Epel, 2005), consistent with simple diffusion. In linear chains of cells, such as trichomes, symplasmic movement is also thought to occur by diffusion (Barclay and Peterson, 1982; Terry and Robards, 1987; Tucker et al., 1989). However, there have been few studies of dye movement at the junction between one symplasmic domain and another, and to our knowledge, there have been no studies in which a single cellular boundary between two symplasmic domains has been probed from opposite sides. Interestingly, in their classical experiments on the *Abutilon* trichome, Terry and Robards (1987) were able to establish that the Stokes radius of a molecule was the major determinant for diffusive movement through PD. Significantly, however, they noted that "no dye, not even the highly mobile LYCH, was able to move from the basal trichome cell into the underlying sepal tissue." This is a striking observation, because this interface contains one of the highest densities of PD reported for plant cells (about 13 per μm^2 of cell wall; Gunning and Hughes, 1976). Terry and Robards (1987) suggested that the lack of dye movement was due to the presence of a symplasmic barrier at the trichome base. However, such a barrier would appear to negate the high symplasmic fluxes of solutes essential to carry prenectar into the *Abutilon* trichomes (Findlay and Mercer, 1971; Gunning and Hughes, 1976). These facts, coupled to the observation that the basal stalk cell of the *Abutilon* trichome contains a suberized apoplastic barrier (Gunning and Hughes, 1976), indicate that solute movement into the trichomes occurs exclusively by symplasmic transport. In the case of the tobacco trichome, it appears that all interconnected trichome cells behave as a single symplasmic domain (Waigmann and Zambryski, 1995), with control of solute movement being exerted at the e/t interface. Interestingly, early last century, Schumacher (1936) reported the polar movement of fluorescein in trichomes of *Cucurbita*, and other authors subsequently championed the concept of polar solute movement through PD (Arisz, 1969). Our data suggest that polar movement through PD may be more common than previously thought, and new studies are now required to test this notion.

We found that unidirectional transport of solutes occurred in both the secreting and nonsecreting trichomes of tobacco. In the latter case, it seems unlikely that mass flow of solutes would have occurred in the absence of secretion (Gunning and Hughes, 1976). When either LYCH or GFP was introduced into trichome cells (other than the basal cell), these probes moved bidirectionally to adjacent trichome cells. If bulk flow of solutes was occurring through the chain of trichome cells, these probes should have moved preferentially in the apical direction toward the site of secretion. The rapid basipetal movement of dyes seen following injection of the apical trichome cell (Oparka and Prior, 1992) and the basipetal movement of fluorescein described here for capped trichomes would also argue against bulk flow. We suggest, instead, that the observed unidirectional flux of solutes is generated specifically at the e/t interface. Movement of both small fluorescent dyes and GFP specifically across the e/t interface by carrier-mediated transmembrane transport is unlikely given the nonspecific movement of solute species we observed. Also, the presence of an apoplastic dye barrier at the e/t interface appears to preclude the apoplastic transport of solutes. Thus, the PD at the e/t boundary are the most likely candidates for solute movement into the trichome. If movement of membrane-impermeant probes through PD had occurred by uniform diffusion, we would have expected dyes introduced into the basal trichome cell cytoplasm to have moved freely into the epidermis. However, we observed an almost complete barrier to basipetal movement across the e/t boundary.

The continuous movement of solutes into the trichome via the symplasm could be explained if incoming solutes were compartmentalized (e.g. by delivery into the vacuole) or were rapidly metabolized, both of which are likely in leaf trichomes specialized for the synthesis of defense compounds. However, the unidirectional transport of solutes poses water relations problems, as the continuous entry of solutes into the trichome would be accompanied by movement of water from the mesophyll. In the case of secreting trichomes, the buildup in turgor pressure would be dissipated during the continuous release of exudate across the cuticle of the terminal secretory cell. However, unexpectedly, we also found unidirectional transport into nonsecretory trichomes. This poses the question of how such cells avoid the buildup of turgor pressure, as water cannot leave these cells unless it actively recycles back into the mesophyll after solutes have been compartmentalized within the trichome. Clearly, the water relations of the trichome deserve further study in connection with the observations raised here.

TMV MP has been observed to increase the SEL of PD (Wolf et al., 1989). GFP did not cross the e/t barrier in trichomes during a natural TMV infection or in tobacco plants transgenically expressing viral MP, similar to our observations of noninfected trichomes. Furthermore, apical movement of GFP in these trichomes was dramatically reduced relative to noninfected trichomes. This result is consistent with the observations of Guenoune-Gelbart et al. (2008), who suggested that viral MP may dilate the central desmotubule of PD, restricting the cytoplasmic channel available for cell-to-cell transport. Our finding that viral infection does not influence solute movement out

of the trichome into the epidermis is consistent with the recent finding of Christensen et al. (2009) that GFP does not exit the basal cell of tobacco trichomes during movement of the TMV genome, and it is also consistent with the view that the PD at the e/t boundary do not display the "classic" gating phenomenon reported for mesophyll cells (Waigmann and Zambryski, 2000; Christensen et al., 2009).

The basal barrier to symplasmic solute exit was overcome completely by sodium azide, allowing LYCH to move out of the trichome. The extensive movement we observed in the presence of azide is compatible with simple diffusion and would be predicted if active solute movement was eliminated at the e/t boundary. Metabolic inhibitors have been shown previously to increase the SEL of PD and also to cause lateral solute leakage out of the transport phloem (Wright and Oparka, 1997). These observations indicate that at least some aspects of PD function may be under strict metabolic control. We suggest that solute directionality, in addition to PD SEL, may be under active control and that some PD may be involved in the directional movement of solutes into a symplasmic domain.

The current views of PD structure/function are somewhat contradictory. On the one hand, PD are viewed as simple pores whose permeability is governed by diffusion (Liarzi and Epel, 2005), and on the other hand, they are viewed as complex structures that can facilitate selective trafficking of macromolecules (Lucas and Lee, 2004), features that are sometimes difficult to reconcile. However, it is clear that PD have a complex substructure (Roberts, 2005). For example, "sphincters" have been observed in the wall around the neck region of PD (Olesen, 1979), and connections have been observed between the desmotubule and PM at the neck region of the pore (Badelt et al., 1994). In addition, unique globular proteins are associated with both the outer membrane of the desmotubule and the PM (Ding et al., 1992), while radially arranged "spokes" appear to connect the desmotubule to the PM along the length of the pore (Maule, 2008). Cytoskeletal elements have also been localized to PD, particularly proteins associated with actin/myosin-based motility (for review, see Aaziz et al., 2001; Oparka, 2004). In mesophyll cells of tobacco, inhibition of the actin cytoskeleton was shown to increase the SEL of PD (Ding et al., 1996), and actin has been immunolocalized to PD (White et al., 1994). Unfortunately, the proteomics of PD has proved difficult for technical reasons, and only a few bona fide PD proteins have been identified to date (Maule, 2008). Until the full repertoire of PD proteins is revealed, the functions of PD-specific proteins will remain a mystery.

In our experiments, actin depolymerization of the trichome cytoskeleton failed to allow leakage of injected dyes out of the tobacco trichome, suggesting that trichome PD display fundamental differences to mesophyll PD in both structure and function (Waigmann and Zambryski, 2000). Substructures within the PD pore are usually viewed as "skeletal" and are thought to be involved in maintaining pore aperture and hence SEL (Blackman et al., 1999; Oparka, 2004). It is conceivable that some of these structures may also play a role in unidirectional solute transport through the pore when circumstances require. The plant body is replete with specialized interfaces that are connected by PD, and yet these cells and/or tissues maintain highly autonomous functions (Duckett et al., 1994; Volk et al., 1996; Oparka and Turgeon, 1999; Roberts et al., 2001; Ruan et al., 2001; Ormenese et al., 2002; Facchini et al., 2004). It will be interesting to determine if unidirectional transport through PD is a general feature of those cells that display marked divisions of labor. Functional studies of the PD at these boundaries may provide further insights into the complex functions of PD. The use of the tools described here, particularly the transgenic expression of photoactivatable proteins, may accelerate this analysis.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* 'Xanthi NN', 'Samsun', Xanthi expressing a translational fusion of MP::GFP, and Samsun expressing PAGFP) plants were grown from seed in a heated glasshouse and used for experiments between 20 and 60 d old. Samsun expressing PAGFP was made by leaf disc transformation (Horsh et al., 1985) of Samsun with *pBIBHyg.35S::PAGFP. PAGFP* was blunt cloned into pBIBHyg (Becker, 1990) downstream of a cauliflower mosaic virus 35S promoter. Virus infection was carried out as described by Christensen et al. (2009) using a TMV vector expressing a DsRed reporter.

Confocal Microscopy

The tissue was imaged using a Leica SP2 confocal laser scanning microscope with either a 20× or a 40× water-dipping lens (Leica Microsystems). Calcofluor white was excited at 405 nm, LYCH at 458 nm, GFP and FDA at 488 nm, and propidium iodide at 561 nm. PAGFP was activated at 405 nm.

Microinjection

The micromanipulation equipment was that described by Oparka et al. (1991) attached to a Leica SP2 confocal laser scanning microscope fitted with a 20× long-distance objective, with which all injections were conducted. Microinjection was performed using a modified pressure probe (Oparka and Prior, 1992). Micropipettes were pulled from filamented glass tubes (GC 150F-10; Clark Electromedical) using a Narishige PB-7 micropipette puller and were loaded with dyes through the capillary prior to backfilling with silicone oil. The pressure in the tip of the micropipette tip was adjusted to that of the cells to be impaled. This method minimizes damage due to the loss of cell turgor and does not disrupt cytoplasmic streaming (Oparka and Prior, 1992). LYCH was made up as a stock solution of 500 mM and used at a concentration of 3 mM.

Trichome Capping

FDA (5 μg mL^{-1}) was capillary loaded into CustomTip Type 1 (Eppendorf) microcapillaries. The microcapillary was inserted into the micromanipulator, and the tip of the capillary was placed over the tip cell of a secreting trichome for 10 to 15 min to allow the dye to be taken up into the symplasm.

Photoactivation of PAGFP

PAGFP was activated by three consecutive scans of the nucleus of the cell of interest with the 405-nm laser at 100% power. For trichome cells, the

activated zone was separated from cell walls that join neighboring cells to ensure that GFP fluorescence originated from one cell only. For epidermal cells, the activated zone was spatially separated from the wall of the adjoining trichome, although activation may have occurred in neighboring epidermal cells. Fluorescence in neighboring cells was scored if the nucleus was visible in these cells.

Pressure Probe Measurements

The turgor of basal trichome cells and epidermal supporting cells was measured with a modified pressure probe as described by Oparka et al. (1991).

Application of Inhibitors

To disrupt the actin cytoskeleton, Samsun leaves were infiltrated with 25 μM latrunculin (Calbiochem; 5 mM stock solution in dimethyl sulfoxide) for 15 min. Before injection, trichomes were checked for inhibition of cytoplasmic streaming. Sodium azide (0.02%, 3 mM; VWR International; 10% stock in water) was infiltrated into Samsun leaves and incubated for 20 min before injection.

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